Using EGFP fusions to monitor the functional expression of GPCRs in the *Drosophila* Schneider 2 cells

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Abstract In combining fluorescence measurements with ligand binding assays, the versatility of the EGFP C-terminally fused to the human mu opioid receptor (EGFP-hMOR) has been exploited to notably improve the expression level of functional G protein-coupled receptors in *Drosophila* S2 cells. A selected array of efficient optimization approaches is presented herein, ranging from a cell-sorting method, allowing for a substantial enrichment in EGFP-hMOR expressing cells, to the addition of chemical and pharmacological chaperones, significantly enhancing the yield and the activity of the expressed receptors. Consistent with previous studies, significant discrepancies were

observed between the total amounts of fluorescent receptors over a limited subpopulation capable of ligand binding, even after expression optimization. Subsequently, membrane isopycnic centrifugation experiments allowed to separate the ligand binding active from the non-active membrane fraction, the latter most probably containing misfolded receptors. Taken together, these results illustrate a coherent set of advantageous productive and preparative methods for the production of GPCRs in the highly valuable *Drosophila* S2 expression system.

Keywords *Drosophila* S2 cells · Fluorescence · G protein-coupled receptor · Heterologous expression · Human mu opioid receptor · Isopycnic centrifugation · Ligand binding · Optimization

Abbreviations

DMSO	Dimethyl sulfoxide
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
GPCR	G protein-coupled receptor
hMOR	Human mu opioid receptor
PBS	Phosphate-buffered saline
S2 cells	<i>Drosophila</i> Schneider 2 cells

Introduction

About only 5% of the existing G protein-coupled receptors (GPCRs) represent the primary target of more than 30% of the drugs on the market today

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(Schlyer and Horuk 2006; Hopkins and Groom 2002). These figures reflect the enormous potential for the pharmaceutical industry to exploit this increasingly important family of proteins. However, despite the huge efforts employed in this direction, current conventional approaches have proven far less efficient in bringing further classes of GPCR-targeted drugs through the pipeline (Terstappen and Reggiani 2001). As an alternative of choice, structure-based drug design represents a highly promising way to uncover new lead compounds (Cavasotto and Orry 2007), provided that exploitable structural information is available. While only two tertiary structures of GPCRs have been reported so far (Palczewski et al. 2000; Cherezov et al. 2007), there is a pressing demand for other GPCR structural data. Efficient and accurate expression systems allowing for such studies are even more requested.

We have shown in previous studies how the *Drosophila melanogaster* Schneider 2 cells (S2 cells) could be a highly potential and scaleable system for the production of a functionally active GPCR, namely the human mu opioid receptor N-terminally fused to the enhanced green fluorescent protein (EGFP-hMOR) (Perret et al. 2003; Brillet et al. 2006). This receptor was stably expressed to higher levels compared to a similar stable Sf9 recombinant cell line (Kempf et al. 2002), exhibiting a pharmacological profile close to what was reported for expression in mammalian cell lines and bearing an effective coupling to the endogenous $G_{i/o}$ protein (Perret et al. 2003). Furthermore, it was demonstrated that large-scale production of hMOR was also achievable using recombinant S2 cells cultured in a bioreactor (Brillet et al. 2006). For structural studies however, these different data suggested that further optimization was needed for improving both the yields and the functional homogeneity of the produced receptors. To this end, various strategies were envisioned that had already proven successful with other expression systems. Apart from sequence modification approaches, several expression optimization studies conducted with bacterial (Weiss and Grisshammer 2002; Stanasila et al. 1999), yeast (Andre et al. 2006; Grunewald et al. 2004; Sarraemgna et al. 2002) and higher eukaryotic host systems (Hassaine et al. 2006; Akermoun et al. 2005; Massotte et al. 1999) showed that expression levels of functional GPCRs could be substantially improved by tuning some external growth factors such as

temperature and time of induction, cell densities, formulation of growth media or supplementation with stabilizing compounds or chemical chaperones. The goal of the present study is to explore several of these routes in order to optimize the production of functional GPCRs expressed in a recombinant Schneider S2 system. Utilizing the hMOR-EGFP fusion construct as a prototype, fluorescence measurements, combined to specific ligand binding data, were used to assess the effect of a panel of methodologies on the yield and functionality of the expressed receptors.

Materials and methods

Cell culture and induction

The human mu opioid receptor (hMOR) fused to the enhanced green fluorescent protein (EGFP) was cloned into the pMT/BiP vector under the control of the CuSO_4 inducible metallothionein promoter and then stably introduced into *Drosophila melanogaster* Schneider 2 cells (Invitrogen) as previously described (Perret et al. 2003). The stably transfected cell line was grown at 27 °C in Insect X Press medium (Cambrex Biosciences, Verviers, Belgium) supplemented with 0.2% pluronic F-68, 50 $\mu\text{g mL}^{-1}$ gentamicin (Invitrogen) and 10% FBS (Invitrogen) which was heat inactivated at 65 °C for 30 min. Cell concentration was determined by viable cell counting (trypan blue exclusion) in a hemacytometer.

Kinetic experiments for EGFP-hMOR expression were performed upon 700 μM CuSO_4 induction. When mentioned, the cells were supplemented overnight with 10 μM Naloxone (Sigma-Aldrich, St. Louis, MO, USA), and/or 2.5–5% DMSO (Sigma-Aldrich, St. Louis, MO, USA).

Magnetic cell sorting using goat anti-mouse IgG microbeads

After CuSO_4 induction, about 10^7 cells were pelleted at 900 rpm for 3 min and resuspended in 25 μL PEB buffer (PBS, 5 mM EDTA, 1% BSA). This cell suspension was incubated for 10 min at 4 °C with PEB buffer supplemented with 1/200 anti-EGFP monoclonal antibody (Boehringer Ingelheim, Mannheim, Germany), then washed twice with PEB buffer,

resuspended in 25 μ L PEB buffer supplemented with 1/5 goat anti-mouse IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) before incubation for 15 min at 4 °C. The cell suspension was then washed with PEB buffer, centrifuged 900 rpm 10 min and resuspended in PEB buffer. The MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) was fixed onto its magnet and the cell suspension was loaded. The column was washed three times with PEB buffer. The column was then removed from the magnet and the EGFP expressing cells were eluted with 1 mL of PEB buffer. The sorted out cells were washed with Insect X Press medium and split in 12 wells plates.

Ligand binding assays

Induced cells were centrifuged at 1,000 rpm for 3 min at 4 °C, washed twice in PBS buffer supplemented with 320 mM sucrose and resuspended in 50 mM Tris–HCl pH 7.4, 320 mM sucrose. Saturation ligand binding assays were performed using 2×10^6 cells in a total volume of 0.5 mL, in the presence of [3 H]-diprenorphine ranging from 0.05 to 3.2 nM (NEN Life Sciences Products, Boston, MA, USA). After 40 min incubation at room temperature, triplicate samples were filtered on GF/B filters, pretreated with 0.1% polyethylenimine (Sigma Aldrich, St. Louis, MO, USA) and washed twice with ice-cold 50 mM Tris pH 7.4, using a Brandel cell harvester (Semat International, St. Albans, England). The non-specific binding was determined in the same conditions using 2 μ M Naloxone (Sigma Aldrich, St. Louis, MO, USA) as a competitor. The binding data were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Fluorescence measurements

EGFP fluorescence measurements were made in Insect Elliot Buffer (5.44 mM KCl, 129.7 mM NaCl, 1.2 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 7.3 mM NaHCO_3 , 20 mM Hepes, 63 mM sucrose, pH 6.8) using a dedicated spectrofluorimeter (Photon Technology International) with slits sets to yield a bandwidth of 2 nm at excitation and emission. The cell suspensions (10^7 cells/mL) were placed in a 1 mL cuvette with magnetic stirring and maintained at 21 °C in a thermostated cell handler.

After excitation at 470 nm, the EGFP fluorescence spectrum was registered from 490 to 750 nm. The specific fluorescence of the sample at 510 nm was then calculated after withdrawing the background fluorescence determined from non-transfected S2 cells.

Membrane separation by isopycnic centrifugation on a sucrose gradient

The membrane separation was performed by floatation on a discontinuous sucrose gradient. A frozen pellet of EGFP-hMOR cells was resuspended in 50 mM Tris pH 7.4, 4 mM EDTA, 250 mM sucrose, 1 mM PMSF (2.10^8 cells mL^{-1}). The cell suspension was then sonicated and centrifuged for 3 min at 1,000 rpm, 4 °C. The post-nuclear supernatant was collected and brought to a final concentration of 1.5 M sucrose. A sucrose discontinuous gradient was achieved in Beckman SW41 tubes loaded with 3 mL of membrane preparation in 1.5 M sucrose, overlaid with 6.5 mL of 1.2 M sucrose, 50 mM Tris pH 7.4, 4 mM EDTA, and 3.5 mL of a third layer of 0.8 M sucrose, 50 mM Tris pH 7.4, 4 mM EDTA. After a 20 h centrifugation at 35,000 rpm, at 4 °C, fractions (500 μ L) were collected from the top (sample 1) to the bottom (sample 27) of the gradient and analysed through fluorescence measurements and ligand binding assays.

Western blot analysis

Samples were mixed with a Laemmli buffer for 30 min at room temperature and then loaded onto a 10% SDS–polyacrylamide gel. After SDS-PAGE, proteins were electrotransferred onto a PVDF membrane. Following a blocking step in 3% nonfat dried milk in PBS 0.5% Tween 20 (MPBST) for 30 min, membranes were incubated for 1 h in MPBST supplemented with 1/1,000 anti-EGFP monoclonal (Boehringer Ingelheim, Mannheim, Germany). Membranes were washed three times in MPBST and then incubated in MPBST supplemented with 1/2,000 anti-mouse IgG HRP-linked whole antibody from sheep (Amersham) for 1 h. After four washes with MPBST, chemiluminescence was obtained and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Results

Enrichment of EGFP-hMOR expressing cell populations through magnetic cell sorting

In agreement with the expression variability that is often reported with this system, EGFP-hMOR was not homogeneously produced upon induction in the *Drosophila* S2 cell populations (Perret et al. 2003), and about only 40% of these cells were observed to express the fluorescent fusion under a Zeiss Axiovert 135 fluorescence microscope. Thus, as a first step to improve the receptor expression levels, several methods were carried out to select the best producing cell sub-populations. While classical limiting dilution approaches did not lead to a significant increase of the overall expression levels (data not shown), fluorescent activated cell sorting (FACS) techniques allowed for a notable enrichment in detected receptors but were hampered with repeated cell contamination problems. We therefore preferred to focus on an alternative immunogenic magnetic cell sorting technology using MACS[®] beads (Miltenyi Biotec) for enriching this polyclonal cell line with the best expressing cells. As shown in Fig. 1a, the fluorescence signal of EGFP-hMOR detected after separation on MACS columns was about twice higher compared to the non-sorted S2 cells, with up to 80% of cells expressing the fluorescent fusion as assessed by flow cytometry (data not shown). Similarly, saturation ligand binding experiments showed a comparable improvement of the receptor activity, with an increase of specific binding values from about 14,000 to 21,000 receptors per cell before and after magnetic cell sorting, respectively (Fig. 1b).

Improvement of expression levels through media supplementation with exogenous chaperones

Among the different experimental parameters that have been reported to affect the production levels of recombinant proteins, some are more related to the host system while others depend mostly on the type of protein to be produced. Regarding the former category, we showed in a previous study (Perret et al. 2003) an increase of EGFP-hMOR expression with time, reaching a maximum production level after 72 h induction. Comparable observations were obtained separately for

Fig. 1 (a) Fluorescence measurements of non transfected S2 cells (◆), induced EGFP-hMOR recombinant cells (□) and induced EGFP-hMOR cells sorted out with MACS columns (■). (b) Total ligand binding sites of induced EGFP-hMOR (gray bar) and induced EGFP-hMOR sorted cells (black bar)

two chemokine GPCRs, CCR5 and CXCR4, similarly expressed as EGFP fusions in S2 cells (unpublished data). We also observed that an optimization in the inducer CuSO₄ concentration could slightly modulate the expression level with an optimal efficiency measured at 700 μM CuSO₄.

With the aim of screening for external factors that would act more specifically on the receptor in order to improve the production yield and/or functionality, exogenous chaperones were added in the media during the induction phase. As an illustration of the positive effect that could be obtained with such chemical chaperones, Fig. 2 shows the ligand binding activity of the EGFP-hMOR fusion that was determined in a time

Fig. 2 Time course expression of EGFP-hMOR in S2 cells: ligand binding measurements in standard induction conditions (gray bars) versus induction in presence of 10 μ M naloxone (black bars)

course experiment with naloxone, a specific opioid receptor antagonist, added during the induction phase. In the presence of 10 μ M naloxone, the total amount of binding sites was already three times higher than in standard conditions after 24 h induction, with a maximal activity measured 72 h post-induction.

In another set of experiments, both the fluorescence and the ligand binding activity of EGFP-hMOR produced in S2 cells were determined after 72 h induction, in the presence of various concentrations of dimethyl sulfoxide (DMSO) and/or 10 μ M naloxone. The obtained values were then divided by the same data measured in the standard condition in order to evaluate the relative evolution of the receptor yields (fluorescence ratio) and activity (ligand binding ratio) upon the tested conditions (Fig. 3). As was previously observed for S2 cells induced in presence of 10 μ M naloxone, the ligand binding activity was three fold higher while the total yield was kept at the same level. Interestingly, an increasing concentration of DMSO from 2.5 to 5% had a dramatic effect on the expression levels since both the ligand binding (activity) and fluorescence (yields) were increased up to 10 fold compared to the standard condition. Finally, a combined addition of both 10 μ M naloxone and 2.5% DMSO resulted in an apparent cumulative effect of the two chemical chaperones.

Fig. 3 Improvement factor of EGFP-hMOR expression assessed by fluorescence measurements (gray bars) and binding assays (black bars) in presence of naloxone and various concentration of DMSO. The induction was performed for 72 h with 700 μ M CuSO_4

Assessing the best expression conditions as a function of optimal receptor yield and activity

The previous representation illustrates the improvement factor of total receptor yield and the corresponding binding activity that could be achieved upon different induction conditions. With the aim of producing a given GPCR for structural studies, the next step is to identify the best induction condition that would allow for the highest yield of functional receptor. To this end, the EGFP fluorescence was used to quantify the total expression levels of EGFP-hMOR in the cell using a calibration curve obtained with purified EGFP as previously described (Perret et al. 2003). The resulting values (Table 1) were then compared to the number of functional receptors assessed via ligand binding, and the corresponding percentage of functional amongst total EGFP-hMOR was determined (Table 1, last column). Regarding the conditions in which the highest yield could be attained, the addition of 4% DMSO was by far the most interesting (more than 650,000 fluorescent receptors per cell). However, less than 7% of these receptors were identified as active in a ligand binding assay. In this regard, the addition of naloxone was significantly more beneficial for restoring the functional property of these receptors, The combination of 10 μ M

Table 1 Comparison of ligand binding and fluorescent values obtained from the EGFP-hMOR receptors expressed in different induction conditions

Induction conditions	Specific fluorescence (counts/s)	Total fluorescent receptors per cell	Total ligand binding receptors per cell	Ligand binding versus fluorescent receptors (%)
Standard	30,220	81,120	3,392	4.2
+10 μ M Naloxone	27,894	75,844	10,658	14.0
+2.5% DMSO	89,929	216,556	10,638	4.9
+3% DMSO	130,560	308,717	13,690	4.4
+4% DMSO	282,020	652,268	44,362	6.8
+5% DMSO	224,690	522,229	30,712	5.9
+2.5% DMSO + naloxone	101,290	242,325	35,851	14.8

The total amounts of fluorescent receptors were calculated from a fluorescence calibration curve obtained with a soluble EGFP as described in Perret et al. (2003)

naloxone and 2.5% DMSO added during induction led to the best compromise since about 15% of ligand binding active EGFP-hMOR could be detected among a total of 240,000 fluorescent receptors per cell.

In all cases, these results highlighted a strong discrepancy between total versus functional amounts of receptors expressed in the S2 cells. We showed in a previous study that a significant portion of EGFP-hMOR was retained within the cells (Perret et al. 2003). Consequently, internal functional receptors might not be detected through a classical ligand binding assay on whole cells, and the overall activity could be therefore underestimated. Alternatively, the produced EGFP-hMOR might embrace different conformations pending on their location within internal or plasma membranes, potentially affecting their functionality and the globally detected ligand binding activity. In order to assay these hypotheses, membranes of S2 cells were fractionated and further analysed.

Distribution of functional EGFP-hMOR among subcellular membranes

After cell lysis, post-nuclear supernatants were subjected to an isopycnic centrifugation on a three-step sucrose gradient. In these conditions, two membrane populations could be isolated: a light (0.8 and 1.2 M sucrose interface) and a heavy (1.2 and 1.5 M sucrose interface) fraction, containing plasma membranes and the Golgi complex (light membranes), and the rough endoplasmic reticulum (heavy membranes), respectively. The presence of the fluorescent EGFP-hMOR

fusions could be strikingly visualized in both light and heavy membrane fractions when placing the tube under a UV lamp after centrifugation. In order to analyse the distribution and the activity of the receptors within these membranes, 27 fractions of 500 μ L were collected from the top of the tube and were analyzed through fluorescence measurements and ligand binding assays. From the obtained data, the relative distribution of receptor yield and activity among the 27 fractions was calculated and therefore expressed as a percentage of the globally detected signal (Fig. 4). As previously observed, the fluorescent material was partitioned between both light (samples 4–9) and heavy (samples 19–27) membranes, with about 70% of the signal detected in the latter fractions. On the other hand, the ligand binding activity was found to be quite exclusively present within the light membrane fractions, suggesting that the larger portion of non active EGFP-hMOR fusions previously observed mainly corresponded to receptors stacked in heavy membrane internal compartments. A complementary anti-EGFP Western blot analysis was performed on the same samples (Fig. 5), leading to the specific detection of the EGFP-hMOR receptor with a similar distribution between light and heavy membranes. Furthermore, this experiment revealed a differential migration between the two types of samples since additional bands of higher molecular size were identified in the heavy fractions, suggesting that receptors present in the heavy membranes could be stacked as oligomers or aggregates.

Taken together, these results emphasize the importance of this preparative method for enriching S2 membranes with functional receptors.

Fig. 4 EGFP-hMOR partition in S2 cell membranes fractionated by a sucrose floatation gradient. Percentages of the global fluorescence (*black bars*) and ligand binding (*gray bars*) were determined for each of the 27 fractions collected from the top of the gradient

Fig. 5 Western blot analysis of the gradient sucrose fractions: EGFP-hMOR was specifically detected using a monoclonal anti-EGFP antibody (1/1,000 dilution). White arrow indicates the band of the expected size for a EGFP-hMOR monomer

Discussion

It has been shown in a previous study how the use of an EGFP-fused GPCR expressed in *Drosophila melanogaster* S2 cells could be a very attractive, valuable and versatile system for the production of functional receptors (Perret et al. 2003). In order to make it suitable for the important yields of active receptors required for structural studies, this work concluded that the system had to be optimized and that large culturing procedures were to be developed. While this latter point was successfully addressed in a dedicated paper describing how recombinant S2 cells could be easily and advantageously cultured using scalable bioreactors (Brillet et al. 2006), we have explored some of the routes that could be envisioned to here optimize the expression yields and functionality of EGFP-fused receptors.

Consistent with previous observations for various heterologous proteins (Yokomizo et al. 2007; Santos et al. 2007; Perret et al. 2003), recombinant S2 cells

were heterogeneously expressing EGFP-hMOR fusions. Thus, as a first improvement step, cell sorting techniques were assayed for enriching the polyclonal S2 cell lines in receptor-expressing sub-populations. In doing so, we found that the MACS[®] immunogenic cell sorting method (Miltenyi Biotec) was very convenient and efficient that allowed to double the number of cells expressing functional receptors as assessed through fluorescence and ligand binding measurements. A similar finding was also observed with another recombinant S2 cell line expressing an EGFP-fused CCR5 chemokine receptor, subjected to cell sorting using this technique (data not shown). However, after several passages of both cell lines, these significant improvements in fluorescence and ligand binding signals were further observed to decrease back to the initial levels or even lower, meaning that the enrichment in EGFP-GPCR expressing cells was efficient but reversible. Further investigations are needed in order to precisely evaluate this peculiar behaviour of recombinant S2 cells during GPCR expression and to suggest

possible explanations and respective solutions. Meanwhile, cell sorting is required to be performed repeatedly and we found that magnetic cell sorting, being an efficient, easy-to-handle and cost-effective method, is particularly well suited for this purpose.

Another way of choice for improving the yield of functional GPCRs produced in a recombinant system is to appropriately adjust some of the experimental parameters that influence the host cell physiology and hence its performance for heterologous gene expression, correct protein folding and proper trafficking. Several studies have reported on such optimization, focusing on vector backbones and fusion sequences, induction temperature, time course and media composition (for a review, see Sarraemegna et al. 2003), even though a few of them were related to the *Drosophila* S2 system (Perret et al. 2003; Tota et al. 1995).

An increasing number of examples also show that adding small molecules facilitates the folding and processing of proteins and might be a general phenomenon that could be applied to a large variety of proteins and conditions (see Bernier et al. 2004). Such small molecules can be divided into two classes: the chemical chaperones, which act as generic stabilizers of folding intermediates for all proteins, and the pharmacological chaperones, which show selectivity of action by binding and stabilizing only the targeted protein. In the present study, supplementation of the induction media with a chemical chaperone such as dimethylsulfoxide (DMSO) or with naloxone, a receptor-specific antagonist compound acting as a pharmacological chaperone, proved to substantially increase both the amount and the binding activity of the EGFP-hMOR fusion produced in S2 cells. Furthermore, a cooperative effect was observed when a combination of both compounds was added to the medium. When compared to similar studies conducted on several GPCRs with other expression systems, where it was shown that the main effect of these chaperones was to improve the functionality of the expressed receptors (André et al. 2006; Shukla et al. 2006; Sarraemegna et al. 2002), our results unexpectedly demonstrated that these molecules also increased the total amount of EGFP-hMOR. Whether this particular feature is related to the S2 cells system alone or is receptor dependent needs to be further investigated.

Regardless of the expression system in use, only a fraction of the expressed GPCRs is capable of ligand binding, a common feature observed in all studies,

including the present one. In an attempt to better characterize the large portion of non-active EGFP-hMOR detected in the S2 cells, and with the aim of separating these receptors from the active ones, membranes were fractionated upon a two-step floatation gradient. Impressively, the light and heavy membranes that were recovered this way revealed a very contrastive content since all the ligand binding activity was measured in the former fraction while the larger non-active population of receptors was detected in the latter one. Furthermore, a Western blot analysis revealed that samples corresponding to heavy membranes containing the non-active receptors presented a migration pattern typical of GPCR multimers or aggregates. Even if deeper investigations are required to properly characterize the subcellular membranes present in these two types of fractions, one can anticipate that the light one would contain plasma and Golgi membranes, whereas the heavy fraction would comprise membranes from the endoplasmic reticulum, according to the literature (Subhashri and Shaila 2007; Frangioni et al. 1992). Thus, we can speculate that the correctly folded and active EGFP-hMOR receptors would most probably enter the trafficking pathway up to the plasma membrane. The misfolded non-active receptors, on the other hand, would be sorted and stacked as multimers into subcellular compartments in a quality control mechanism similar to already described processes (Conn et al. 2007; Sitia and Braakman 2003). Further investigating this process would be highly valuable for a better understanding of the rationale beyond GPCR folding or misfolding when overexpressed in S2 cells. Nonetheless, the two-step floatation gradient we used turned out to be a rapid and efficient method for preparing membrane fractions enriched with functional EGFP-hMOR receptors, arising in this regard as a very useful optimization step.

Overall, a coherent set of methods was developed that allow for significantly improving the amounts of active receptors expressed in the *Drosophila* S2 cells, giving rise to the highly critical quantity and quality of GPCR material required for structural studies.

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